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## Physiological Stress Responses in Strains of the Gilthead Sea Bream (*Sparus aurata*)

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### Abstract

In this study we examined the physiological responses of different strains of gilthead sea bream (*Sparus aurata*) to chronic and acute stress factors. Blood glucose levels, hematocrit, and lactate concentrations were examined in yearlings of different strains of gilthead sea bream subjected to different stress situations. In the first experiment, fish of the Ardag and 'ebony' strains were kept 45 days in moderate (35-45 kg/m<sup>3</sup>) or high (70-90 kg/m<sup>3</sup>) density. There were no significant effects on the blood glucose level but, at both densities, hematocrit was significantly higher in the Ardag strain (33.2±3.0% at moderate and 44.5±1.6% at high) than in the ebony strain (22.0±3.0% and 19.9±2.7%, respectively). The interaction of 'strain' and 'crowding' was highly significant, suggesting that hematocrit may respond differently in each strain to crowding. In the second experiment, we examined the effects of acute stress (handling without anesthesia) among four strains representing Mendelian mutations that affect body coloration in gilthead sea bream: ebony, 'white', 'yellow', and the normally pigmented Ardag. Significant variations in the glucose and lactate concentrations suggest that genotypes of gilthead sea bream may have heritable differences in their physiological responses to stress factors.

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## Introduction

The gilthead sea bream (*Sparus aurata*) is one of the most important cultured marine fish in countries surrounding the Mediterranean Sea. Since 1990, the main Israeli commercial culture of this species was concentrated in industrial sea cage farms in the Gulf of Eilat. However, in 2008, all sea cage mariculture activity was terminated due to governmental regulations. It is anticipated that future development of Israeli mariculture will be in desert areas of the Negev and Arava, in land-based intensive recirculating seawater or brackish water aquaculture systems (RAS; Gordin, 2003). In Israel, marine species are cultured in environments ranging from extensive ponds (low densities of 10-15 kg fish/m<sup>3</sup>) to semi-intensive raceways and cages (moderate densities of 15-25 kg fish/m<sup>3</sup>) to highly intensive facilities (high and very high densities of 50-120 kg fish/m<sup>3</sup>) such as the new pilot RAS in Eilat (Noam Mozes, pers. comm.)

In addition to high fish density (or 'crowding'), intensive culture is associated with handling, grading, transportation, and other chronic or acute disturbances that can induce stress. Culture of fish on farms is an industrial process and acute or chronic stress situations are part of the regular routine procedures (Schreck, 1981). Such stress situations and factors are also unavoidable for captive cultured fish. Jointly or separately, stress factors can significantly affect fish culture performance, i.e., external appearance, survival, disease resistance, and growth rate (Barton and Iwama, 1991; Pickering, 1992). The immune system of animals exposed to physical disturbances such as handling and severe crowding may be compromised, affecting their well-being and ability to resist disease (Schreck, 1981).

At the National Center for Mariculture (NCM) on the Red Sea in Eilat, Israel, the selective breeding program has developed several genetically improved strains of gilthead sea bream which have better growth and survival in the industrial sea cage environment (Knibb, 2000; Gorshkov, 2006). However, a breeding program for the development of improved gilthead sea bream strains adapted for an intensive land-based aquaculture industry in the Arava and Negev is needed. Physiological stress responses in cultured freshwater channel catfish (*Ictalurus punctatus*) and rainbow trout (*Oncorhynchus mykiss*) are associated with the genetic background of the strains used in the experiment (Pottinger and Pickering, 1997; Bosworth et al., 2004; Weber and Silverstein, 2007). Therefore, selective breeding programs for gilthead sea bream should aim at developing strains with stress responses better suited to intensive rearing conditions. Little is known about variations in the physiological stress responses of different sea bream strains, yet simply choosing a strain(s) that is better suited to stress conditions in intensive culture could be equivalent to years of selection of genetically inferior strains (Kinghorn, 1983).

The objective of our work was to characterize the variation in physiological responses to chronic and acute stress factors in different strains and genetic groups of gilthead sea bream by measuring three blood components - glucose, lactate, and hematocrit - that are considered indicators of physiological stress response.

## Materials and Methods

*Experimental fish and rearing conditions.* Two experiments were conducted at the NCM using immature sea bream reared at the NCM and the RAS in Eilat. The fish were from different strains, each with more than 15 years of culture history (see Knibb, 2000, and Gorshkov, 2006, for details on development of the strains and their role in Israeli mariculture). One of the strains was a domesticated crossbred and normally pigmented fish transferred from a local industrial fish farm called Ardag. This strain originated from our long-term program of mass selection and crossbreeding for improved growth. The other strains represented Mendelian mutations of sea bream that affect body coloration and show discrete patterns of inheritance and pleiotropic effects: 'ebony', 'yellow', and 'white'. These mutations were developed at the NCM and, as unique genetic resources, can be used in basic fish genetics and mariculture research (Gorshkov, 2006).

The fish were acclimated for 30 days prior to the experiments. They were kept indoors in circular flow-through plastic tanks of 80-l capacity (0.4 m depth), supplied with a constant flow of sea water at 480 l/h (40-41 ppt salinity, 5.6 mg/l aeration,  $24 \pm 0.5^\circ\text{C}$ , natural photoperiod). Fish were fed commercial dry pellets (Matmor, M.P. Evtach, Israel) daily at a rate of 1.2% body weight. The fish were not fed one day before sampling.

*The first experiment - chronic stress.* In the first experiment, we examined physiological stress responses that could possibly be attributed to genetic make-up. To test these responses, the Ardag and ebony strains were exposed to a crowding factor ('density rearing'). Two rearing densities were tested: moderate ( $35\text{--}45 \text{ kg/m}^3$ ) and high ( $70\text{--}90 \text{ kg/m}^3$ ). To simulate the moderate density, 120 Ardag and 120 ebony individuals were divided equally and randomly stocked into three tanks for each strain (40 fish x six tanks). We used the same number of Ardag and ebony to obtain the high density, but adjusted the heights of the external stand-pipes so that there was only half the quantity of water in the 80-l tanks. The fish were left undisturbed for 45 days before blood sampling, when they weighed a mean of  $89.5 \pm 21.1 \text{ g}$ .

*The second experiment - acute stress.* In the second experiment we examined the effects of acute handling stress on the physiological stress responses in Ardag and three strains of Mendelian color mutations (ebony, yellow, and white). The fish ( $160.3 \pm 45.8 \text{ g}$  mean wt) were kept in duplicate tanks at a density of  $70\text{--}90 \text{ kg/m}^3$ . They were exposed to conditions simulating acute handling stress by being chased with a net for 1 min, netting five fish from the tank and holding them in the air for 30 s, then transferring them to a 20-l tank with a water level of about 8-10 cm so that they could swim only on their sides for 6-7 min prior to blood sampling.

*Sampling.* Five fish from each replicate tank were removed from the holding tanks and blood samples were taken within 30 sec (3-4 min for the five fish sampled from each tank). One ml blood was collected from the caudal vein of each specimen using a 21-gauge needle and 1-ml heparin-coated syringes. Aliquots of 20  $\mu\text{l}$  of fresh blood were used for glucose and lactate analyses and blood was introduced into heparinized microhematocrit

tubes for calculating the percent hematocrit. The rest of the sample was allowed to clot at 4°C for 6 h and then, following centrifugation, the serum was removed and frozen at -20°C until required. After bleeding, fish were killed, dissected, and weighed to the nearest 1 g. Although five fish were bled in each sampling, there were occasions when no more than 1 ml of blood was obtained from an individual fish, especially of the ebony strain.

**Stress indicators.** Samples of 0.003-0.004 ml fresh blood were used to measure glucose and lactate. Glucose levels were determined with a FreeStyle Freedom blood glucose meter (Geffen Medical, Israel) based on an enzymatic colorimetric procedure to a precision of  $\pm 1.8$  mg/dl and variability from strip to strip about of 5.6%. Lactate levels were determined with a Lactate Pro blood lactate test strip meter (ARKRAY, Inc., Japan) based on an amperometric method using an enzymatic reaction to a precision of  $\pm 0.07$  mmol/l and variability from strip to strip of about 3.2%. Hematocrit was determined by centrifuging the blood at 10,000  $\times g$  for 6 min in heparinized microhematocrit tubes and calculating the percentage of erythrocytes.

**Statistical analyses.** All results are presented as means $\pm$ SD of the original untransformed data. As there were no significant variations between replicates, data from replicates were pooled for further analysis. One-way ANOVA was performed to examine the effect of crowding and acute stress on each blood component; if significance differences were found, means were compared using Duncan's multiple range tests to find particular differences between groups. Two-way ANOVA was applied when two factors were treated together as the main factor. In all tests, differences were accepted as significant when  $p < 0.05$ . Statistical tests were performed using SPSS 5.0.1 for Windows (SPSS, Inc., Chicago, Illinois, USA).

## Results

**Effect of chronic stress on blood components.** The blood glucose levels ranged from  $68.6 \pm 6.6$  to  $76.3 \pm 10.2$  mg/dl and there were no significant differences ( $p > 0.05$ ) between fish kept at moderate and high densities, or between the Ardag and ebony stains. Hematocrit values in the Ardag strain were significantly higher than those in the ebony strain at both moderate ( $34.2 \pm 3.0\%$  vs  $22.0 \pm 3.0\%$ , respectively) and high ( $44.5 \pm 1.6\%$  vs  $19.9 \pm 2.7\%$ ) densities. In addition, Ardag kept at the moderate density had a significantly ( $p = 0.006$ ) lower hematocrit concentration than Ardag kept in high density. Two-way ANOVA indicated statistically significant effects of strain, density, and their interaction on the hematocrit concentrations (Table 1). The  $p$  value for the factor 'density' was 0.03, meaning that the effect of density was not as strong as that of 'strain' ( $p < 0.001$ ). The interaction of the two factors was highly statistically significant ( $p = 0.004$ ), indicating that the two strains respond differently to the density factor.

**Effect of acute stress on blood components.** In the second experiment, there were significant differences in glucose and lactate levels between the tested strains (Table 2). Blood glucose was significantly higher in the white strain, while lactate was significantly higher in ebony. Hematocrit concentration was similar in all strains.

Table 1. Two-way analysis of variance of the hematocrit concentrations (%) in Ardag and ebony strains of gilthead sea bream kept in moderate and high densities.

Source of variation	d.f.	SS	MS	F ratio	P value
Strain	1	1012.00	1012.00	146.00	0.001**
Density	1	49.60	49.60	7.20	0.03*
Strain x density	1	115.32	115.32	16.60	0.004**
Residual	8	55.4	6.95	-	-

d.f. = degree of freedom

SS = sum of squares

MS = mean square = SS/d.f.

F = the ratio between variation within a group to variation between groups

P = probability value

\* significant ( $p < 0.05$ )

\*\* highly significant ( $p < 0.01$ )

Table 2. Mean blood components in four strains of gilthead sea bream exposed to acute stress (n = 10 from duplicate tanks).

Blood component	Strain			
	Ardag	Ebony	Yellow	White
Glucose (mg/dl)	66.8±10.8 <sup>b</sup>	52.8±16.1 <sup>b</sup>	49.0±15.5 <sup>b</sup>	100.2±22.9 <sup>a</sup>
Lactate (mmol/l)	2.5±0.5 <sup>b</sup>	5.6±2.1 <sup>a</sup>	3.5±1.0 <sup>b</sup>	2.4±0.3 <sup>b</sup>
Hematocrit (%)	40.7±2.5	38.9±17.7	49.5±8.2	33.4±10.3

Values within a row bearing common letters do not significantly differ ( $p > 0.05$ ; Duncan's multiple range test).

## Discussion

Stress is an unavoidable component of any intensive aquaculture where fish are routinely chased, netted, handled, graded, transported, and subjected to other manipulation. Stress impacts range from small and non visible effects, to substantial increases in respiration and blood pressure, decreased reproductive performance, and increased susceptibility to disease that can lead to death (Schreck, 1981).

In our experiments, the baseline values of blood components varied within the relatively wide ranges of 49-100 mg/dl for glucose, 2.4-5.6 mmol/L for lactate, and 20-50% for hematocrit, similar to those observed in other Sparidae (Rotllant and Tort, 1997; Bressler and Ron, 2004; Fanouraki et al., 2007).

Our results suggest that, in the Ardag strain, the hematocrit level, but not the glucose concentration, can serve as an indicator of stress resulting from prolonged rearing at high density (also called chronic stress caused by crowding). There was a similar lack of glucose response in red porgy (*Pagrus pagrus*, Sparidae) subjected to chronic stress by crowding for three weeks (Rotllant and Tort, 1997; Fanouraki et al., 2007). Our results showed significant differences in hematocrit values between the Ardag and ebony

strains, suggesting variation in genetic make-up for physiological stress response. Heritable differences in physiological stress responses were identified by plasma cortisol measuring in cultured strains of rainbow trout (Weber and Silverstein, 2007). In our experiments, the influence of density was not as pronounced as the influence of strain, and we obtained a consistent response to chronic crowding stress in terms of hematocrit concentrations. Simulated chronic crowding had a similar effect on blood parameters in red porgy (Fanouraki et al., 2007) and gilthead sea bream fingerlings (Montero et al. 1999).

Acute stress (netting without anesthesia) resulted in significantly different blood component values among the genetic groups, suggesting that genotypes might have heritable differences in physiological response to acute stress. When another population of gilthead sea bream was netted without anesthetics, glucose levels also rose (Rotllant et al., 2001). Overall, our data demonstrates differences in physiological responses between genetic groups exposed to both crowding and acute stress. However, there was substantial variation in the lactate and hematocrit concentrations among fish of the ebony strain and some of the ebony fish were difficult to bleed, suggesting a higher blood coagulation rate as their specific physiological response to the stressors.

Variation of physiological responses among genetic groups of sea bream is similar to findings in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) where physiological responses (also measured in blood parameters) differed significantly among cultured strains (Fevolden et al., 1991; Weber and Silverstein, 2007). Even a short inspection of published studies shows variability in the levels of main physiological parameters under stress conditions in cultured fish. Much of the variability can be ascribed to differences in experimental feeding regimes, sampling methodology, fish acclimation, or effects of anesthetics (Barton and Iwama, 1991; Bressler and Ron, 2004). However, even in the few cases in which physiological responses of different fish strains to stressors were examined, differences between genetic groups were revealed (Pickering and Pottinger, 1989; McGreer et al., 1991; Pottinger et al., 1992). Similar results were obtained in poultry selected for high and low responsiveness to stress. Chicken strains selected for high responsiveness tend to be more susceptible to viral infections and less susceptible to bacterial infections than unselected birds (Freeman, 1976). Thus, experiments with different cultured animals suggest that their sensitivity to common forms of stress has a genetic background. Stress response may also be influenced by the level of domestication or genetic background of the cultured species.

The mariculture industries in Mediterranean countries are currently working with many different sea bream populations. There is a lack of data on their inherent stress resistance. Identifying populations (or strains) that have greater resistance to stressors has great practical value. The use of different genotypes (strains, lines, or crossbreeds) in intensive rearing systems should be considered in breeding and selection plans as practical ways to obtain fish that are better adapted and have greater resistance to very high densities, and are more robust and capable of handling stress. The improvement of

stress resistance in gilthead sea bream through selective breeding is very important for the development and progress of land-based seawater and brackish-water Israeli aquaculture in the Negev and Arava. The results of this study can facilitate testing, findings, and selecting of available strains or genotypes of gilthead sea bream and other cultured species that perform better in an RAS environment.

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